Published in final edited form as:

Biochem Biophys Res Commun. 2008 October 24; 375(3): 292–296. doi:10.1016/j.bbrc.2008.07.059.

TNF- α upregulates the A_{2B} adenosine receptor gene: the role of NAD(P)H oxidase 4

Cynthia St. Hilaire^{1,2}, Milka Koupenova¹, Shannon H. Carroll¹, Barbara D. Smith¹, and Katya Ravid^{1*}

¹Department of Biochemistry and the Whitaker Cardiovascular Institute, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118

Abstract

Proliferation of vascular smooth muscle cells (VSMC), oxidative stress and elevated inflammatory cytokines are some of the components that contribute to plaque formation in the vasculature. The cytokine tumor necrosis factor- α (TNF- α) is released during vascular injury, and contributes to lesion formation by affecting VSMC proliferation. Recently, an A_{2B} adenosine receptor (A_{2B}AR) knockout mouse illustrated that this receptor is a tissue protector, in that it inhibits VSMC proliferation and attenuates the inflammatory response, including the release of TNF- α . Here, we show a regulatory loop by which TNF- α upregulates the A_{2B}AR in VSMC *in vitro* and *in vivo*. The effect of this cytokine is mimicked by its known downstream target, NAD(P)H oxidase 4 (Nox4). Nox4 upregulates the A_{2B}AR, and Nox inhibitors dampen the effect of TNF- α . Hence, our study is the first to show that signaling associated with vascular disease, such as Nox4, is also able to upregulate the tissue protecting A_{2B}AR.

Keywords

A_{2B} adenosine receptor; vascular smooth muscle cells; tumor necrosis factor- α ; NAD(P)H oxidase 4

INTRODUCTION

Tumor necrosis factor- α (TNF- α) is a potent pro-inflammatory cytokine generated by many types of cells in response to inflammation. TNF- α contributes to atherosclerotic lesion formation, in part by stimulating VSMC to proliferate [1]. Adenosine is a potent autocrine and paracrine signaling molecule generated or released from cells under stress [2], and has been shown to reduce the release of TNF- α from cells stimulated by an immune response [3,4], as its signaling is generally tissue protective [5]. Adenosine elicits its effects through interaction with one or more of the four adenosine receptors: A₁AR, A₃AR, A_{2A}AR and A_{2B}AR [6,7].

Inhibitory effects of adenosine on TNF- α release at baseline or in response to bacterial lipopolysaccharide (LPS) are modulated via the A₂ARs [8,9], and furthermore, in a regulatory

*Corresponding author: Katya Ravid, DSc/PhD, Department of Biochemistry, K225, Boston University School of Medicine, Boston, MA 02118, Tel: 617-638-5053, Fax: 617-638-5339, ravid@biochem.bumc.bu.edu.

²Present address: Laboratory for Comparative Carcinogenesis, National Cancer Institute - Frederick, Bldg 538, Room 144, Frederick, MD, 21702, USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

loop fashion, A_{2B}AR gene expression is upregulated by TNF- α signaling [10–12]. The specific molecules involved in the signaling downstream of TNF- α that induce A_{2B}AR upregulation are unknown. Adenosine signaling through the A_{2B}AR has been shown to inhibit smooth muscle cell proliferation [13,14], and stimulation of A_{2B}AR post-infarction prevents remodeling of cardiac tissues [15]. Thus, as TNF- α is upregulated in atherosclerotic plaques and can induce expression of the A_{2B}AR, and adenosine is released from cells under stress [1,2,5,11,12] and can inhibit VSMC proliferation [13,14], we sought to understand the signaling pathway involved in TNF- α -induced upregulation of A_{2B}AR in the context of VSMC.

One signaling mechanism downstream of TNF- α binding its receptor is the generation of reactive oxygen species (ROS) [16]. In the vasculature, NAD(P)H oxidase (Nox) enzymes are responsible for the majority of ROS production [17], and in VSMC Nox4 seems to be the dominant isoform expressed [18]. Recently, ROS generated by Nox4 were found to be required for VSMC to maintain their fully differentiated phenotype [19]. Nox complexes are made up of multiple proteins, consisting of heme redox components, a 6 transmembrane protein with a carboxy terminus that binds FAD and NAD(P)H moieties to generate ROS [20], and a second membrane-bound component, p22^{phox} [21,22]. TNF- α stimulation was shown to upregulate p22^{phox} in rat VSMC [23], and this component is required for Nox4 activity [24]. In addition, TNF- α specifically upregulates Nox4 in human aortic SMC and the authors suggest that Nox4 is the cause of ROS in the context of inflammatory conditions [25].

In this paper, we sought to elucidate the mechanism of TNF- α -induced upregulation of the A_{2B}AR and propose that the generation of ROS by TNF- α -activated Nox4 regulates factors that induce expression of the A_{2B}AR.

MATERIAL & METHODS

Vascular smooth muscle cell culture conditions

Aortic VSMC were isolated from neonatal Sprague-Dawley rats, as described previously [26,27], and were seeded at a density of 1.5×10^5 cells per well of a 6-well plate; all experiments were performed with cells in their first passage. VSMC were cultured in VSMC growth medium containing DMEM, 10% bovine calf serum (BCS), 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 U/mL penicillin, and 100 μ g/mL streptomycin (all from Invitrogen, Carlsbad, CA).

Measurement of A_{2B}AR mRNA expression

Total RNA from aortic VSMC was prepared with Trizol® (Invitrogen, Carlsbad, Ca) according to the manufacturer's instructions [28]. One μ g of RNA was used to make complementary DNA (cDNA), using the Superscript II kit (Invitrogen, Carlsbad, CA, cat# 18064-22) according to manufacturer's instructions. A_{2B}AR mRNA was quantified using ABI Gene Array TaqMan® primers (ABI, Foster City, CA, cat# Rn00567696_m1), and 18s rRNA (cat# 4319413E) with the TaqMan® Gene Expression Master Mix (ABI, Foster City, CA, cat# 4370048), using the ABI 7300 Real-Time PCR System. In experiments with inhibitors, cells were treated with the inhibitor at the indicated concentrations, with reapplication 24 hours later. When inhibitors were used in conjunction with another stimulus (Ad- β -gal, Ad-Nox4, Ad-DN-Nox4 adenoviruses, or TNF- α), inhibitors were incubated with cells for 1 hour before the stimulus was added.

Adenoviral transduction

Adenoviruses Ad- β -gal, Ad-NAD(P)H oxidase 4 (Nox4) and Ad-DN-Nox4 were a generous gift from Dr. Barry Goldstein, Thomas Jefferson University [29], and were used at a multiplicity of infection (MOI) of 50. In promoter construct transfection assays, adenoviral

particles were added first and 3–6 hours later, the medium was replaced, followed by DNA transfection as described below.

Human growth hormone quantification

Three to four days post-transfection of plasmid using FuGENE 6®, 200 L of media was collected and quantification of human growth hormone (hGH) secreted into media was determined using the hGH ELISA kit® (Roche, Indianapolis, IN cat# 1585878) according to manufacturer's instructions. Absorbance was read with a µQuant microplate reader (Bio-Tek Instruments, Winooski, VT).

β-Galactosidase enzymatic assay

β-Galactosidase (β-gal) assays were performed to quantify the amount of enzyme in the aorta of A_{2B}AR-knockout/β-gal-knock-in (A_{2B}AR-β-gal) mice [27]. Organs were snap frozen, ground and directly mixed with PMI solution (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgCl₂, 50 mM β-mercaptoethanol (American Bioanalytical, Natick, MA, cat# AB01340) and 0.2 mg 2-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma-Aldrich, St. Louis, MO, cat# N1127)) at a concentration of 150 µl PMI per 3 mg of protein. The solution was incubated in a 37°C water bath for 1–3 hours and stopped by addition of 0.5 mL 1M Na₂CO₃. Solution was analyzed on a spectrophotometer at 420 nm (O.D. typically in the range of 0.2 to 0.7).

In vivo TNF-α injection into mice

All mice used were bred in our facilities or purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6J wild-type mice (cat# 000664) were used as controls against the A_{2B}AR-β-gal knockout mice. Mice were injected intraperitoneally with 150 µg/kg of recombinant mouse TNF-α and 16 hours after injection organs were isolated and subjected to β-galactosidase enzymatic activity assay, as described above, or subjected to RNA preparation.

Statistical analysis

Statistical significance was determined by a two-tailed Student's t-test for unpaired variables assuming equal variance. Data were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Effect of an inflammation-inducing agent and TNF-α on A_{2B}AR mRNA and promoter activity *in vitro* and *in vivo*

In addition to our findings describing the inducibility of the A_{2B}AR gene in proliferating VSMC by the transcription factor B-Myb [30], a recent paper by Kong et al. identified HIF-1α as responsible for the induction of A_{2B}AR expression in endothelial cells under hypoxic conditions [31]. In the context of atherosclerosis, proliferation and hypoxia are stress responses, and A_{2B}AR activation has been shown to be protective in these instances [14,31]. Inflammation also plays a role in atherosclerosis [32], and as the A_{2B}AR has been shown to also have a protective effect in regard to inflammatory stress [33], we sought to investigate the influence of the inflammatory response on A_{2B}AR gene expression. This was pursued by first using bacterial lipopolysaccharide (LPS) as an inflammation-inducing agent [34]. Figure 1A illustrates that LPS significantly increases A_{2B}AR mRNA levels. LPS binds to toll-like receptors and elicits a signaling pathway that causes the release of inflammatory cytokines, such as TNF-α [27]. As shown in Figure 1B, TNF-α mimicked the effects seen with LPS. To investigate the A_{2B}AR gene promoter activity directly, -5.81-hGH [30] was transfected into VSMC and effects of LPS and TNF-α were quantified. The A_{2B}AR gene promoter activity was similarly increased when cells were treated with LPS and TNF-α (Figure 1C).

TNF- α upregulation of A_{2B}AR mRNA *in vivo*

To examine the above effect *in vivo*, we utilized the A_{2B}AR- β -gal mouse, in which the endogenous A_{2B}AR gene promoter is driving the gene encoding for β -galactosidase (β -gal) [27]. According to previous results, the expression of the A_{2B}AR gene in the vasculature is uneven, with very few patches in the aorta and a greater abundance in the mesenteric artery [27]. To determine if TNF- α upregulates A_{2B}AR expression *in vivo*, mice were injected with recombinant mouse TNF- α , and 16 hours later the artery was isolated and protein lysate subjected to an assay for β -gal activity. Figure 2A illustrates that TNF- α significantly increases the endogenous A_{2B}AR gene promoter by nearly 1.5-fold *in vivo*. Similarly, wild-type mice injected with TNF- α showed a significant induction of A_{2B}AR mRNA in the artery, matching the expression pattern seen in the A_{2B}AR- β -gal mouse (Figure 2B). These results show that the *in vitro* effects seen with A_{2B}AR expression in cultured vascular smooth muscle cells and promoter construct activity are mimicked in the context of the mouse *in vivo*.

Nox4 can mimic the effect of TNF- α on A_{2B}AR mRNA and promoter activity

One downstream effect of TNF- α binding to its receptor on vascular smooth muscle cells is the activation and upregulation of NAD(P)H oxidase 4 (Nox4) [25]. In VSMC, Nox4 activity can be increased upon stimulation [17]. Consistent with previously published findings, exposure to TNF- α increased levels of ROS in VSMC [35], and addition of H₂O₂ alone induced activation of the A_{2B}AR gene promoter (Supplementary Figure 1A & B). To determine if Nox4 can mimic the effect of TNF- α , we used an adenovirus to over-express Nox4 [29] in primary VSMC. Overexpression of Nox4 significantly increased the level of A_{2B}AR mRNA as compared to control adenovirus (Figure 3).

Additionally we used an adenovirus that over-expresses a dominant-negative form of Nox4 that lacks the FAD binding domain (DN-Nox4) [29]. As compared to the wild-type, the dominant-negative form does not induce expression of A_{2B}AR mRNA. Additionally, A_{2B}AR mRNA expression in cells transduced with Ad-DN-Nox4 was significantly lower compared to cells transduced with Ad- β -gal (Figure 3). The data indicate that activity of Nox4 induces A_{2B}AR mRNA expression. When the -5.81-hGH A_{2B}AR gene promoter construct is transfected into VSMC over-expressing Nox4, the promoter activity is increased in the same pattern as that seen at the mRNA level (Supplementary Figure 2). The responses to over-expression of Nox4 mimic those observed with application of TNF- α , suggesting that Nox4 might be a component of the TNF- α -mediated regulation of A_{2B}AR expression.

Effect of Nox inhibitors on A_{2B}AR mRNA

To test if Nox activation mediates TNF- α effects on A_{2B}AR gene expression, we used Nox inhibitors. Apocynin is a plant-derived chemical that functions as an inhibitor of Nox enzyme activity by preventing cytosolic components of the Nox complex from interacting with the membrane-bound components, thus rendering the enzyme inactive [36]. Diphenylene iodonium (DPI) is reduced by the flavin moieties in various ROS-generating enzymes, and subsequently forming a covalent bond with the flavin or adjacent amino acids within the Nox enzyme, thus rendering it inactive [37]. Primary aortic VSMC were pretreated with inhibitors for 1 hour followed by application of TNF- α , as detailed in Materials and Methods. Figure 4 illustrates that both apocynin and DPI reduced TNF- α -induced A_{2B}AR mRNA expression, suggesting that the activation of Nox4 is upstream of A_{2B}AR gene expression in this mechanism.

In vascular injury or in the formation of an atherosclerotic plaque, secretion of TNF- α from leukocytes and macrophages recruited to the inflamed area contributes to lesion formation, in part, by stimulating VSMC to proliferate and migrate into the lumen [1,38,39]. Adenosine is an autocrine and paracrine signaling molecule generated or released from cells under stress

[2]. It has been shown to inhibit the release of TNF- α from cells stimulated by an immune response [3,4], which supports the conclusion that its signaling is generally tissue protective [5]. Adenosine inhibits TNF- α release from monocytes and macrophages by signaling through the A_{2A}AR or A_{2B}AR, both at baseline and in response to bacterial LPS [8,40]. Furthermore, in a regulatory-loop fashion, A_{2B}AR gene expression has been shown to be upregulated by TNF- α in astroglial and intestinal endothelial cells [10,11]. Adenosine signaling through the A_{2B}AR has also been found to inhibit smooth muscle cell proliferation [13,14], and stimulation of A_{2B}AR post-infarction prevents remodeling of cardiac tissues [15].

Considering that TNF- α induces expression of the A_{2B}AR in some cell types [10,11], and adenosine is released from cells under stress [1,2,5], which can inhibit VSMC proliferation [13,14], we examined whether TNF- α affects A_{2B}AR expression in VSMC. We found that TNF- α stimulated A_{2B}AR gene promoter activity and increased mRNA expression in cultured cells (Figure 1), as well as in *in vivo* mouse models (Figure 2).

TNF- α stimulation can generate ROS by activating Nox enzymes [41]. Additionally, TNF- α signaling upregulates *Nox4* gene expression in human aortic SMC, which is responsible for the generation of ROS under inflammatory conditions in these cells [25]. In accordance with this mechanism, when Nox4 was over-expressed, A_{2B}AR mRNA and gene promoter activity were increased (Figure 3). Additionally, inhibitors of Nox activity prevented TNF- α -induced upregulation of A_{2B}AR mRNA (Figure 4).

A study with endothelial cells showed that Nox enzymes are involved in TNF- α -induced expression of E-selectin, VCAM-I, and ICAM-I, which are adhesion molecules that aid in the recruitment of leukocytes to the inflamed or injured area [42]. The A_{2B}AR- β -gal knockout mouse showed an increase in some of these same adhesion molecules [27], suggesting that the A_{2B}AR has effects that oppose those of the TNF- α -induced-Nox activity in the regulation of these adhesion molecules. Future studies will focus on exploring this intriguing regulatory link.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Dr. Barbara Schreiber and the cell core for preparation of smooth muscle cell cells and for insight, as well as Dr. Xu Yong for initial assistance with qPCR. This work was supported by National Institutes of Health (NIH) Public Health Services Grant HL13262. KR is an Established Investigator with the American Heart Association.

REFERENCES

1. Libby P. Inflammation in atherosclerosis. *Nature* 2002;420:868–874. [PubMed: 12490960]
2. Hasko G, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. *TRENDS in Immunology* 2004;25:33–39. [PubMed: 14698282]
3. Hasko G, Kuhel DG, Chen JF, Schwarzschild MA, Deitch EA, Mabley JG, Marton A, Szabo C. Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A2a receptor-dependent and independent mechanisms. *Faseb J* 2000;14:2065–2074. [PubMed: 11023991]
4. Hasko G, Szabo C, Nemeth ZH, Kvetan V, Pastores SM, Vizi ES. Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J Immunol* 1996;157:4634–4640. [PubMed: 8906843]
5. Sitkovsky MV, Ohta A. The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? *Trends Immunol* 2005;26:299–304. [PubMed: 15922945]
6. Obata T. Adenosine production and its interaction with protection of ischemic and reperfusion injury of the myocardium. *Life Sci* 2002;71:2083–2103. [PubMed: 12204768]

7. Linden J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol* 2001;41:775–787. [PubMed: 11264476]
8. Zhang JG, Hepburn L, Cruz G, Borman RA, Clark KL. The role of adenosine A2A and A2B receptors in the regulation of TNF- α production by human monocytes. *Biochemical Pharmacology* 2005;69:883–889. [PubMed: 15748700]
9. Kreckler LM, Wan TC, Ge ZD, Auchampach JA. Adenosine inhibits tumor necrosis factor- α release from mouse peritoneal macrophages via A2A and A2B but not the A3 adenosine receptor. *J Pharmacol Exp Ther* 2006;317:172–180. [PubMed: 16339914]
10. Kolachala V, Asamoah V, Wang L, Obertone TS, Ziegler TR, Merlin D, Sitaraman SV. TNF- α upregulates adenosine 2b (A2b) receptor expression and signaling in intestinal epithelial cells: a basis for A2bR overexpression in colitis. *Cell Mol Life Sci* 2005;62:2647–2657. [PubMed: 16322943]
11. Trincavelli ML, Marroni M, Tuscano D, Ceruti S, Mazzola A, Mitro N, Abbracchio MP, Martini C. Regulation of A2B adenosine receptor functioning by tumour necrosis factor α in human astroglial cells. *J Neurochem* 2004;91:1180–1190. [PubMed: 15569261]
12. Kolachala V, Asamoah V, Wang L, Obertone TS, Ziegler TR, Merlin D, Sitaraman SV. TNF- α upregulates adenosine 2b (A2b) receptor expression and signaling in intestinal epithelial cells: a basis for A2bR overexpression in colitis. *Cellular and Molecular Life Sciences* 2005;62:2647–2657. [PubMed: 16322943]
13. Dubey RK, Gillespie DG, Osaka K, Suzuki F, Jackson EK. Adenosine inhibits growth of rat aortic smooth muscle cells. Possible role of A2b receptor. *Hypertension* 1996;27:786–793. [PubMed: 8613241]
14. Dubey RK, Gillespie DG, Mi Z, Jackson EK. Adenosine inhibits growth of human aortic smooth muscle cells via A2b receptors. *Hypertension* 1998;31:516–521. [PubMed: 9453355]
15. Wakeno M, Minamino T, Seguchi O, Okazaki H, Tsukamoto O, Okada KI, Hirata A, Fujita M, Asanuma H, Kim J, Komamura K, Takashima S, Mochizuki N, Kitakaze M. Long-Term Stimulation of Adenosine A2b Receptors Begun After Myocardial Infarction Prevents Cardiac Remodeling in Rats. *Circulation*. 2006
16. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *Embo J* 1998;17:2596–2606. [PubMed: 9564042]
17. Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* 2003;285:R277–R297. [PubMed: 12855411]
18. Winkler K, Wunsch S, Kreutz R, Rothermund L, Paul M, Schmidt HH. Upregulation of the vascular NAD(P)H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system in vitro and in vivo. *Free Radic Biol Med* 2001;31:1456–1464. [PubMed: 11728818]
19. Clempus RE, Sorescu D, Dikalova AE, Pounkova L, Jo P, Sorescu GP, Schmidt HH, Lassegue B, Griendling KK. Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. *Arterioscler Thromb Vasc Biol* 2007;27:42–48. [PubMed: 17082491]
20. Krause KH. Tissue distribution and putative physiological function of NOX family NADPH oxidases. *Jpn J Infect Dis* 2004;57:S28–S29. [PubMed: 15507765]
21. Leusen JH, Verhoeven AJ, Roos D. Interactions between the components of the human NADPH oxidase: a review about the intrigues in the phox family. *Front Biosci* 1996;1:d72–d90. [PubMed: 9159214]
22. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 2004;4:181–189. [PubMed: 15039755]
23. De Keulenaer GW, Alexander RW, Ushio-Fukai M, Ishizaka N, Griendling KK. Tumour necrosis factor α activates a p22phox-based NADH oxidase in vascular smooth muscle. *Biochem J* 1998;329(Pt 3):653–657. [PubMed: 9445395]
24. Ambasta RK, Kumar P, Griendling KK, Schmidt HH, Busse R, Brandes RP. Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. *J Biol Chem* 2004;279:45935–45941. [PubMed: 15322091]
25. Moe KT, Aulia S, Jiang F, Chua YL, Wong MC, Dusting GJ. Differential upregulation of Nox homologues of NADPH oxidase by tumor necrosis factor- α in human aortic smooth muscle and embryonic kidney cells. *J Cell Mol Med* 2006;10:231–239. [PubMed: 16563235]

26. Zhao Z, Francis CE, Ravid K. An A3-subtype adenosine receptor is highly expressed in rat vascular smooth muscle cells: its role in attenuating adenosine-induced increase in cAMP. *Microvasc Res* 1997;54:243–252. [PubMed: 9441895]
27. Yang D, Zhang Y, Nguyen HG, Koupenova M, Chauhan AK, Makitalo M, Jones MR, St. Hilaire C, Seldin DC, Toselli P, Lamperti E, Schreiber BM, Gavras H, Wagner DD, Ravid K. The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. *J Clin Invest* 2006;116:1913–1923. [PubMed: 16823489]
28. Chomczynski P, Sacchi N. Single Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Analytical Biochemistry* 1987;162:156. [PubMed: 2440339]
29. Mahadev K, Motoshima H, Wu X, Ruddy JM, Arnold RS, Cheng G, Lambeth JD, Goldstein BJ. The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H₂O₂ and plays an integral role in insulin signal transduction. *Mol Cell Biol* 2004;24:1844–1854. [PubMed: 14966267]
30. St. Hilaire C, Yang D, Schreiber BM, Ravid K. B-Myb regulates the A2B adenosine receptor in vascular smooth muscle cells. *Journal of Cellular Biochemistry* 103;2008:1962–1974.
31. Kong T, Westerman KA, Faigle M, Eltzschig HK, Colgan SP. HIF-dependent induction of adenosine A2B receptor in hypoxia. *Faseb J* 2006;20:2242–2250. [PubMed: 17077301]
32. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002;105:1135–1143. [PubMed: 11877368]
33. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 2001;414:916. [PubMed: 11780065]
34. Rietschel ET, Schletter J, Weidemann B, El-Samalouti V, Mattern T, Zahringer U, Seydel U, Brade H, Flad HD, Kusumoto S, Gupta D, Dziarski R, Ulmer AJ. Lipopolysaccharide and peptidoglycan: CD14-dependent bacterial inducers of inflammation. *Microb Drug Resist* 1998;4:37–44. [PubMed: 9533723]
35. Haddad JJ, Land SC. A non-hypoxic, ROS-sensitive pathway mediates TNF- α -dependent regulation of HIF-1 α . *FEBS Lett* 2001;505:269–274. [PubMed: 11566189]
36. Ximenes VF, Kanegae MP, Rissato SR, Galhiane MS. The oxidation of apocynin catalyzed by myeloperoxidase: proposal for NADPH oxidase inhibition. *Arch Biochem Biophys* 2007;457:134–141. [PubMed: 17166480]
37. O'Donnell BV, Tew DG, Jones OT, England PJ. Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochem J* 1993;290(Pt 1):41–49. [PubMed: 8439298]
38. Mattana J, Effiong C, Kapasi A, Singhal PC. Leukocyte-polytetrafluoroethylene interaction enhances proliferation of vascular smooth muscle cells via tumor necrosis factor- α secretion. *Kidney Int* 1997;52:1478–1485. [PubMed: 9407493]
39. Tanaka H, Sukhova G, Schwartz D, Libby P. Proliferating arterial smooth muscle cells after balloon injury express TNF- α but not interleukin-1 or basic fibroblast growth factor. *Arterioscler Thromb Vasc Biol* 1996;16:12–18. [PubMed: 8548411]
40. Kreckler LM, Wan TC, Ge Z-D, Auchampach JA. Adenosine Inhibits Tumor Necrosis Factor- α Release from Mouse Peritoneal Macrophages via A2A and A2B but Not the A3 Adenosine Receptor. *J Pharmacol Exp Ther* 2006;317:172–180. [PubMed: 16339914]
41. Satriano JA, Shuldiner M, Hora K, Xing Y, Shan Z, Schlondorff D. Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor- α and immunoglobulin G. Evidence for involvement of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase. *J Clin Invest* 1993;92:1564–1571. [PubMed: 8397228]
42. Paysant JR, Rupin A, Verbeuren TJ. Effect of NADPH oxidase inhibition on E-selectin expression induced by concomitant anoxia/reoxygenation and TNF- α . *Endothelium* 2002;9:263–271. [PubMed: 12572857]

Figure 1.

Upregulation of A_{2B}AR mRNA in response to inflammatory stimuli. **A.** Rat aortic VSMC were serum starved prior to treatment with vehicle (control) or bacterial lipopolysaccharide (LPS). Total RNA was collected after 48 hours of exposure and subjected to qPCR. Data are expressed as means of three experiments $\pm$ standard deviation (n=3). **B.** VSMC were treated with vehicle (control) or mouse TNF- α (TNF). Total RNA was collected after 48 hours of exposure and subjected to qPCR. Data expressed as means $\pm$ standard deviation (n=3). **C.** VSMC were serum starved and transfected with -5.81-hGH, in which the murine A_{2B}AR gene promoter drives expression of hGH. Vehicle (control), LPS or mouse TNF- α (TNF) was administered at 24-hour intervals, and media were collected for hGH assay 72 hours after transfection. Data are expressed as means $\pm$ standard deviation (n=3) * denotes $p \leq 0.05$ compared to control.

Figure 2.

TNF- α -induced $A_{2B}AR$ mRNA upregulation *in vivo*. Mice were injected with PBS (Vehicle) or 150 g/kg TNF- α (TNF) and organs collected 16 hours post injection. **A.** Mesenteric artery from $A_{2B}AR$ - β -gal mice were snap frozen, pulverized, then subjected to enzymatic β -galactosidase assay. Data are expressed as means $\pm$ standard deviation (n=3). **B.** Mesenteric artery from wild-type mice injected with PBS (control) or 150 μ g/kg TNF- α (TNF) were used for qPCR analysis. Results shown are means (n=12) $\pm$ standard deviation.

Figure 3.

Over-expression of Nox4 upregulates A_{2B}AR mRNA. Primary aortic VSMC were serum starved, then transduced with adenovirus over-expressing NAD(P)H oxidase 4 (Nox4), a dominant-negative Nox4 (DN-Nox4) or control virus (Ad-β-gal). Total RNA was collected after 72 hours and subjected to qPCR. Data are expressed as means ± standard deviation (n=3). * denotes $p \leq 0.01$ compared to Ad-β-gal.

Figure 4.

Inhibition of Nox4 activity prevent TNF- α -induced upregulation of A_{2B}AR mRNA. VSMC were pretreated with vehicle (control), 500 μ M apocynin or 2 μ M DPI for 1 hour followed by addition of 20 ng/mL recombinant mouse TNF- α . The latter was repeated 24 hours later. The concentrations of these inhibitors did not affect cell viability (trypan blue staining; data not shown). Total RNA was collected and subjected to qPCR. Data are expressed as means $\pm$ standard deviation (n=3). * denotes $p \leq 0.007$ compared to control.